Cancer Gene Therapy (2008) 15, 140–153
 0929-1903/08 $30.00
www.nature.com/cgt

ORIGINAL ARTICLE

New highly potent and specific E6 and E7 siRNAs for treatment of HPV16 positive cervical cancer

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Persistent infection by high-risk types of human papillomaviruses (HPV) is a necessary cause of cervical cancer, with HPV16 the most prevalent, accounting for more than 50% of reported cases. The virus encodes the *E6* and *E7* oncoproteins, whose expression is essential for maintenance of the malignant phenotype. To select efficacious siRNAs applicable to RNAi therapy for patients with HPV16+ cervical cancer, E6 and E7 siRNAs were designed using siDirect computer software, after which 10 compatible with all HPV16 variants were selected, and then extensively examined for RNAi activity and specificity using HPV16+ and HPV16− cells. Three siRNAs with the highest RNAi activities toward E6 and E7 expression, as well as specific and potent growth suppression of HPV16+ cancer cells as low as 1 nM were chosen. Growth suppression was accompanied by accumulation of p53 and $p21^{WAF1/CIP1}$, as well as morphological and cytochemical changes characteristic of cellular senescence. Antitumor activity of one of the selected siRNAs was confirmed by retarded tumor growth of HPV16+ cells in NOD/SCID mice when locally injected in a complex with atelocollagen. Our results demonstrate that these E6 and E7 siRNAs are promising therapeutic agents for treatment of virus-related cancer.

Cancer Gene Therapy (2008) **15,**140–153; doi:10.1038/sj.cgt.7701118; published online 21 December 2007

Keywords: siRNA therapy; HPV16; cervical cancer

Introduction

Cervical cancer is the second most common type of cancer encountered in females, with an incidence rate of up to 18.7 per 100 000.[1] Epidemiological and experimental studies have shown that persistent infections with high-risk types of human papillomaviruses (hrHPVs; HPV16, 18, 31, 33, 45) are a necessary cause of cervical cancer, with HPV16 accounting for approximately 50% of reported cases of HPV-related cervical cancer. These viruses encode the *E6* and *E7* oncogenes, whose expression is essential for virus replication.[2–4] Furthermore, deregulated E6 and E7 expression leads to malignant transformation, and is pivotal for maintenance of the malignant phenotype of cervical cancer.

Cellular targets for these hrHPV oncogenes have been investigated, with the p53 tumor suppressor protein the first identified E6 target and still the most important. The E6 protein forms a tertiary complex with E6AP functioning as a ubiquitin ligase and its substrate, p53, which leads to degradation of p53. Other E6 targets include the PDZ family of proteins, such as PSD-95, hDlg and ZO-1, as well as the co-activator p300/CBP. E6 also induces the expression of human telomerase reverse transcriptase (hTERT), a catalytic subunit of human telomerase, whose activity may be involved in immortalization.

Targets of the E7 protein include the pocket protein family of retinoblastoma protein (Rb) and the cyclin-dependent kinase inhibitors $p21^{WAF1/CIP1}$ and $p27^{KIP1}$.[2–4] Since these molecules are negative regulators of the cell cycle, their inhibition by E7 results in upregulation of genes required for G_1/S transition and DNA synthesis, which is essential for viral genome replication and amplification.[5] However, E7 also induces genome instability

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Received 10 September 2007; revised 15 November 2007; accepted 25 November 2007; published online 21 December 2007

and genetic alterations, which subsequently accumulate and cause transformation of infected cells into malignant cells. Recently, E7 was shown to form a complex with promyelocytic leukemia protein (PML), which has been implicated in controlling cellular senescence as a pro-senescence factor.[6]

Because of the strong relationships between the expression of HPV oncogenes and cervical cancer carcinogenesis, several technologies have been applied to target virus oncogenes to develop novel therapies for cervical cancer. Antisense oligo-deoxynucleotides and ribozymes targeting virus oncogenes were found to suppress growth of virus-related cancer cells both *in vitro* and *in vivo*.[7–9] Introduction of a short double-stranded RNA with a duplex region of 19 base pairs and two nucleotide 3′ overhangs, known as a short-interfering RNA (siRNA), induces sequence-specific destruction of endogenous mRNA in mammalian cells, a phenomenon known as RNA interference (RNAi). Since the discovery of siRNA-induced RNAi in 2001,[10,11] siRNAs have been utilized in reverse genetics and gene-targeting therapy. siRNA technology is completely different from others, in that siRNAs utilizes endogenous RNAi machineries that exert normal cellular functions. Studies have shown that introduction of those targeting E6 and E7 of hrHPVs resulted in growth suppression of virus-positive cancer cells.[12–19] These results indicate the possibility of applying RNAi technology to therapeutic options for HPV + cancer.

Although siRNA-induced target gene suppression was initially assumed to be highly sequence specific, it has become clear that siRNAs suppress unintended genes through RNA-induced silencing complex (RISC)-mediated cleavage of target mRNA or translation suppression, which is analogous to miRNA-mediated RNAi,[20–22] and stimulation of innate immune by type I interferon (IFN-I) and inflammatory cytokine responses.[23,24] Suppression of such unintended genes by siRNAs is known as an off-target effect. RISC-mediated off-target effects are caused by a partial sequence complementarity between the guide strand and unintended genes, especially when excessive RISC is present.[25] Furthermore, some siRNAs have been reported to induce innate immune responses in a sequence-specific manner.[23,24] For the purpose of applying siRNA technology to therapy for hrHPV-related cancers, it is mandatory to select siRNA sequences targeting E6 and E7 that have high levels of RNAi activity and minimal off-target effects. However, to date, no known HPV16 E6 or E7 siRNA sequence has been analyzed in detail in regard to those factors.

For the present study, we selected siRNA sequences targeting mRNA coding HPV16 E6 and E7 using siDirect software, based on guidelines established by Ui-Tei *et al*.[26,27] From those findings, we developed highly effective siRNA sequences with maximum target specificity, and examined the siRNAs for RNAi activity and specificity using HPV16 + and HPV16− cancer cells. Our results identified three E6 and E7 siRNAs compatible with most HPV16 variants, which were potent and specific in suppression of E6 and E7 expression, as well as growth of HPV16 + cancer cells.

Materials and methods

Cell lines and plasmid transfection

SiHa (HPV16 + cervical cancer), CaSki (HPV16 + cervical cancer), HeLa (HPV18 + cervical cancer) and SK-OV-3 (HPV− ovarian cancer) cell lines were purchased from American Type Culture Collection (ATCC, Rockville, MD). SiHa, CaSki and HeLa cells were maintained in DMEM containing 10% fetal calf serum (FCS), while SK-OV-3 cells were cultured in RPMI1640 containing 10% FCS. The incubations were performed at 37 °C in an atmosphere of 5% CO_2. Plasmid transfection was performed using Lipofectamine 2000 transfection reagent (Invitrogen Corporation, Carlsbad, CA) according to the manufacturer's instructions.

siRNAs and transfection

The siRNAs used in this study were as follows: control siRNA passenger strand, 5′-GUACCGCACGUCAUUC GUAUC-3′; control siRNA guide strand, 5′-UACGAAU GACGUGCGGUACGU-3′; FLuc siRNA passenger strand, 5′-CCGUGGUGUUCGUGUCUAAGA-3′; FLuc siRNA guide strand, 5′-UUAGACACGAACACCACG GUA-3′; lamin A/C siRNA passenger strand, 5′-CU GAAAGCGCGCAAUACCAAG-3′; lamin A/C siRNA guide strand, 5′-UGGUAUUGCGCGCUUUCAGC U-3′; 222 passenger strand,[12] 5′-GAGGUAUAUGACU UUGCUUUU-3′; 222 guide strand, 5′-AAGCAAAGU CAUAUACCUCAC-3′; 660 passenger strand,[12] 5′-AGG AGGAUGAAAUAGAUGGUC-3′; 660 guide strand, 5′-CCAUCUAUUUCAUCCUCCUCC-3′; 375 passenger strand,[14] 5′-UACAACAAACCGUUGUGUGAU-3′; 375 guide strand, 5′-CACACAACGGUUUGUUGUAU U-3′; 186 passenger strand,[13] 5′-GAAUGUGUGUACU GCAAGCAA-3′; 186 guide strand 5′-GCUUGCAGUA CACACAUUCUA-3′; 203 passenger strand,[16] 5′-GCAA CAGUUACUGCGACGUGA-3′; 203 guide strand, 5′-A CGUCGCAGUAACUGUUGCUU-3′; 535 passenger strand,[16] 5′-CACGUAGAGAAACCCAGCUGU-3′; and 535 guide strand, 5′-AGCUGGGUUUCUCUACGU GUU-3′.

The sequences of siRNAs 233, 243, 244, 493, 497, 501, 573, 698, 707 and 752 are shown in Table 1. Twenty-one-base ribonucleotides were synthesized, annealed in buffer (100 mM potassium acetate, 30 mM HEPES-KOH at pH 7.4, 2 mM magnesium acetate) and column-purified (Proligo Co. Ltd, Boulder, CO).

One day before transfection, the cells were plated at a density of 5×10^4 to 1×10^5 cells per well in a six-well plate containing 2 ml of Dulbecco's modified Eagle's medium (DMEM) with 10% FCS. Various amounts of the siRNA were added with Opti-MEM I reduced serum medium (Invitrogen Corporation) up to 50 μl. For SiHa cells, SK-OV-3 cells, and their derivatives, 1.6 μl of Lipofectamine 2000 was diluted with Opti-MEM I reduced serum medium to 50 μl. For HeLa cells and their derivatives, 0.4 μl of Lipofectamine 2000 was diluted with Opti-MEM I reduced serum medium to 50 μl. Diluted siRNA and Lipofectamine 2000 were combined, incubated at room temperature for 20 min and then added to a 2-ml

Table 1 siDirect-selected siRNAs targeting HPV16 E6 and E7

siRNA	*Nucleotide position*	*Mismatch tolerance*	*Sequence (upper, passenger strand; lower, guide strands)*	*Incompatible HPV16 class and subclass*	*Mismatched nucleotide*
117	117–139	3	5′-ACUUUCUGGGUCGCUCCUGUG-3′	EG(131)	131
			5′-CAGGAGCGACCCAGAAAGUUA-3′	Af1	132, 136
				Af2	132
128	128–150	3	5′-CAGAAAGUUACCACAGUUAUG-3′	EG(131)	131
			5′-UAACUGUGGUAACUUUCUGGG-3′	Af1	132, 136, 143, 145
				Af2	132, 143, 145
233	233–255	3	5′-CUUUGCUUUUCGGGAUUUAUG-3′		
			5′-UAAAUCCCGAAAAGCAAAGUC-3′		
243	243–265	3	5′-CGGGAUUUAUGCAUAGUAUAU-3′		
			5′-AUACUAUGCAUAAAUCCCGAA-3′		
244	244–266	2	5′-GGGAUUUAUGCAUAGUAUAUA-3′		
			5′-UAUACUAUGCAUAAAUCCCGA-3′		
324	324–346	3	5′-GAGUAUAGACAUUAUUGUUAU-3′	Af1, Af2, NA1, AA	335
			5′-AACAAUAAUGUCUAUACUCAC-3′		
326	326–348	3	5′-GUAUAGACAUUAUUGUUAUAG-3′	Af1, Af2, NA1, AA	335
			5′-AUAACAAUAAUGUCUAUACUC-3′		
493	493–515	3	5′-GGUGGACCGGUCGAUGUAUGU-3′		
			5′-AUACAUCGACCGGUCCACCGA-3′		
497	497–519	3	5′-GACCGGUCGAUGUAUGUCUUG-3′		
			5′-AGACAUACAUCGACCGGUCCA-3′		
501	501–523	2	5′-GGUCGAUGUAUGUCUUGUUGC-3′		
			5′-AACAAGACAUACAUCGACCGG-3′		
573	573–595	3	5′-CACCUACAUUGCAUGAAUAUA-3′		
			5′-UAUUCAUGCAAUGUAGGUGUA-3′		
583	583–605	3	5′-GCAUGAAUAUAUGUUAGAUUU-3′		
			5′-AUCUAACAUAUAUUCAUGCAA-3′		
615	615–637	3	5′-CAACUGAUCUCUACUGUUAUG-3′	As, Af2	618
			5′-UAACAGUAGAGAUCAGUUGUC-3′		
625	625–647	3	5′-CUACUGUUAUGAGCAAUUAAA-3′		
			5′-UAAUUGCUCAUAACAGUAGAG-3′		
698	698–720	3	5′-CCGGACAGAGCCCAUUACAAU-3′		
			5′-UGUAAUGGGCUCUGUCCGGUU-3′		
707	707–729	3	5′-GCCCAUUACAAUAUUGUAACC-3′		
			5′-UUACAAUAUUGUAAUGGGCUC-3′		
752	752–774	4	5′-CUUCGGUUGUGCGUACAAAGC-3′		
			5′-UUUGUACGCACAACCGAAGCG-3′		

siRNAs were designated by the nucleotide position number of the 3′ end of the guide strand. HPV16 classes and subclasses carrying a mismatch to the guide strand sequence are shown in the column of incompatible HPV16 class and subclass, with mismatched nucleotide positions also shown. The HPV16 classes and subclasses were as follows: Prototype (accession number K02718); European German 131 (EG131; accession number AF536179); East Asian (As; accession number AF534061); African 1 (Af1; accession number AF472508, AF536180); African 2 (Af2; accession number AF472509); Asian-American (AA; accession number AF402678); and North American I (NA1; accession number AF486325).

Underlines indicate an immunostimulatory motif.[24]

culture. The amount of Lipofectamine 2000 was changed in proportion to the culture medium volume. Transfection of siRNAs using Oligofectamine (Invitrogen Corporation) was performed according to the manufacturer's instructions.

Plasmid construction

The HPV16 E6E7 region from nt 231 to 858, ΔNE6E7 (231–858), was obtained by PCR amplification using pSV2-E6E7 (a gift from Dr T Kanda, National Institute for Infectious Disease, Tokyo, Japan)[28] as a template, and E6 sense and E7 antisense primers containing *Not*I sites at the 5′ end were subcloned at the *Not*I site of the psiCheck-2 plasmid, with the resultant plasmid designated as 16NΔE6E7/psiCheck-2. ΔNE6 region (231–559) and E7 region (562–858) were similarly cloned, and inserted between the *Xho*I and *Not*I sites of psiCheck-2, with the resultant plasmids designated as 16ΔNE6/psiCheck-2 and 16E7/psiCheck-2, respectively. An hLuc cDNA fragment isolated from psiCheck-2 was cloned into pcDNA3 at the *Hind*III and *Xba*I sites, and the resultant plasmid was designated as hLuc/pcDNA3. E7 of 16E7/psiCheck-2 was changed to an E7 variant carrying cytosine instead thymine at nt760 using GeneTailor Site-Directed Mutagenesis System (Invitrogen Corporation), and the resultant plasmid was designated as 16E7(760C)/psiCheck-2.

The primers used for plasmid construction were as follows: Not-HPV16_231s, 5′-GCGGCCGCATGACTT

TGCTTTTCGGGAT-3′; Xho-HPV16_231s, 5′-CTCGAG ATGACTTTGCTTTTCGGGAT-3′; Not-HPV16_E6as, 5′-GCGGCCGCTTACAGCTGGGTTTCTCTAC-3′; Xho-HPV16_E7s, 5′-CTCGAGATGCATGGAGATACACC TAC-3′; and Not-HPV16_E7as, 5′-GCGGCCGCTTAT GGTTTCTGAGAACAGA-3′. Underlines indicate the *Not*I and *Xho*I sites.

SiHa, HeLa and SK-OV-3 clones with constitutive expression of firefly luciferase

SiHa, HeLa and SK-OV-3 cells were transfected with a firefly luciferase (Fluc) expression plasmid (hLuc/pcDNA3) using Lipofectamine 2000 according to the manufacturer's instructions, and selected by cultivating in the presence of G418. The resultant stable clones expressing FLuc were designated as FL-SiHa-2, FL-SKOV-5 and FL-HeLa-1, respectively.

Reverse transcription–PCR

Isolation of DNA-free total RNA, synthesis of single-stranded cDNAs and PCR amplification were performed as previously described.[13] cDNA from mock-transfected cells was serially diluted and used as the standard. PCR assays were performed under the following conditions: denaturation at 94 °C for 5 min, followed by 22–26 PCR cycles (denaturation at 94 °C for 30 s, primer-annealing at 58 °C for 30 s, primer-extension at 72 °C for 30 s) and incubation at 72 °C for 5 min. Amplified products were separated on 2.5% agarose gels, stained with SYBR GOLD (Molecular probes, Eugene, OR) and analyzed using an FLA-2000 fluoro-image analyzer (Fuji Film, Tokyo, Japan). Band intensities were quantified using Image Gauge software (Fuji Film), and normalized to those of the β-actin band or those of 18S ribosomal RNA. PCR primers used for detection of E6 and E7 cDNAs are shown in Table 2. Those for detection of β-actin cDNA were as follows: β-actin forward primer, 5′-CTCAC CATGGATGATGATAT-3′ and β-actin reverse primer, 5′-TGGGTCATCTTCTCGCGGTT-3′. A pair of PCR primers for 18S ribosomal RNA was purchased from Applied Biosystems (Foster City, CA).

Immunoblotting

Cells at $2–5 \times 10^5$ were washed with Dulbecco's phosphate-buffered saline (PBS) once, directly dissolved in 100 μl of sodium dodecyl sulfate (SDS)-gel loading buffer and incubated for 5 min at 95 °C. Ten microliters of each sample was then separated on a polyacrylamide gel containing 0.1% SDS. Immunoblot analysis was performed as described previously.[13] Bands were detected using an FLA-2000 fluoro-image analyzer (Fuji Film). Band intensities were quantified using Image Gauge software (Fuji Film) and normalized to those of the lamin A/C band. Anti-p21$^{WAF1/CIP1}$ (clone 70) and anti-lamin A/C monoclonal antibodies (clone 14) were purchased from BD Biosciences (San Jose, CA), while anti-p53 monoclonal antibody (DO-1) came from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

Table 2 PCR primers for HPV16 E6 and E7

PCR primer	*Direction*	*Nucleotide position*	*Sequence*
E6s104	Forward	104–123	5′-ATGTTTCAGGACCCACAGGA-3′
E6as375	Reverse	375–356	5′-TGCTGTTCTAATGTTGTTCC-3′
E6as558	Reverse	558–539	5′-TACAGCTGGGTTTCTCTACG-3′
E7s562	Forward	562–581	5′-ATGCATGGAGATACACCTAC-3′
E7as855	Reverse	855–836	5′-TTATGGTTTCTGAGAACAGA-3′

Analysis of in vitro *cell growth and senescence-associated β-galactosidase staining*

Cell viability was examined with a colorimetric assay using WST-8 (Cell Counting Kit 8, Dojin Laboratories, Kumamoto, Japan) according to the manufacturer's instructions. Senescence-associated β-galactosidase staining was performed using a Senescent Cell Staining Kit (Sigma-Aldrich Corporation, St Louis, MO).

In vivo *tumor growth*

To study the effects of the siRNAs on tumor growth *in vivo*, SiHa cells (2×10^7 cells) were subcutaneously inoculated into 6-week-old NOD/SCID mice, after which tumors slowly formed at the injected site. Six weeks after the inoculation, siRNAs complexed with AteloGene (Koken Co. Ltd, Tokyo, Japan) according to the manufacturer's instructions were directly injected into the tumors at a dose of 500 pmole per tumor every 7 days ($n = 6$ for each group). Thirty-five days after the first siRNA injection, the mice were killed according to the UKCCCR guidelines, and each tumor was excised and weighed. Tumor weight differences were analyzed for significance using a Student's t test, with $P < 0.05$ considered to indicate statistical significance.

Results

Selection of HPV16 E6 and E7 siRNA sequences

A total of 17 siRNA sequences targeting the E6 and E7 coding regions of HPV16 (Table 1, Figure 1a) were selected using siDirect software,[27] which is based on guidelines for designing siRNA sequences for mammalian RNAi.[26] All of the sequences had structural characteristics of functional siRNA[26,30,31] and contained at least three mismatches in the guide strand with the nonredundant sequence set of human genes (mismatch tolerance 3).[27]

Five siRNAs (117, 128, 324, 326, 615) containing nucleotides that differed among HPV16 classes and subclasses were excluded (Table 1, Supplementary Table 1), and a total of 10 siRNAs targeting different regions of E6 and E7 were chosen from the remaining 12 for this study, as follows: 233, 243 and 244, targeting E6-E7 mRNA, but not E6*I-E7 or E6*II-E7 mRNA; 493, 497 and 501, targeting E6-E7 and E6*I-E7 mRNA, but not E6*II-E7 mRNA; 573, 698, 707 and 752, targeting all three splicing variants. After revision of the nonredundant sequence database, two siRNAs (244, 501) were reclassified as mismatch tolerance 2.

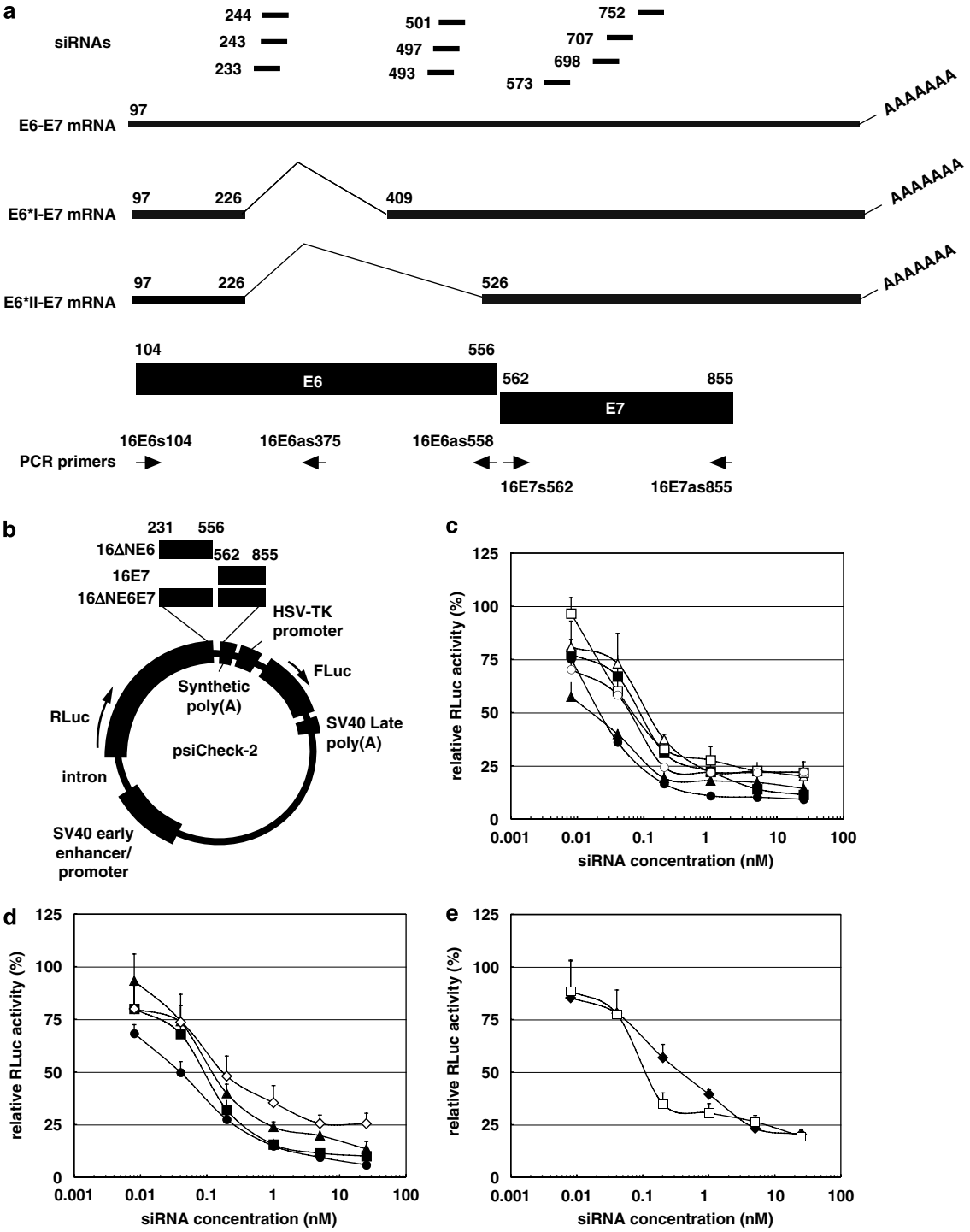


Figure 1 siDirect-selected siRNAs targeting HPV16 E6 and E7, and their effects on luciferase-E6 and -E7 fusion gene expression. (**a**) E6 and E7 siRNAs, mRNA splicing variants, genomic organization and PCR primers. siRNAs targeting E6 and E7 are depicted as short lines (top). Thick bars represent naturally occurring E6E7 mRNA species with introns within E6 (middle). The closed boxes show the locations of the E6 and E7 coding regions of the HPV16 prototype (accession number K02718) (bottom). Positions of the PCR primers used in this study are indicated as arrows. The numbers refer to the locations of the transcription initiating site and splicing junctions.[29] (**b**) Illustration of *Renilla* luciferase (Rluc)-E6E7 fusion gene expression plasmids. SiHa cells were sequentially transfected with an RLuc-ΔNE6E7 (**c**), RLuc-ΔNE6 (**d**) or RLuc-ΔNE6E7 expression plasmid (**e**) and various concentrations of E6 and E7 siRNAs. Forty-eight hours after transfection, the cells were assayed for firefly luciferase (Fluc) and RLuc activities. RLuc activity was normalized to FLuc activity, with RLuc activity of mock-transfected cells defined as 100%. Closed triangle represents siRNA 233; closed square, siRNA 243; closed circle, siRNA 244; open diagonal, siRNA 493; closed diagonal, siRNA 573; open triangle, siRNA 698; open square, siRNA 707 and open circle, siRNA 752. Experiments were performed in triplicate. Error bars represent the s.d. from the mean.

RNAi activities of E6 and E7 siRNAs toward Renilla *luciferase-E6E7 fusion mRNA*

The RNAi activities of the E6 and E7 siRNAs selected using siDirect software were examined with a reporter gene assay. SiHa HPV16+ cancer cells were transfected with a *Renilla* luciferase (RLuc)-ΔNE6E7 fusion gene expression plasmid (Figure 1b), along with various concentrations of the different siRNAs (233, 243, 244, 493, 573, 698, 707, 752). After 48 h of incubation, RLuc and FLuc activities were determined (Figure 1c). The RNAi activities of siRNA 493 and 573 toward RLuc-ΔNE6E7 were low (data not shown), and thus reevaluated using RLuc-ΔNE6 and RLuc-E7, respectively (Figures 1d and e). All the siRNAs examined showed high RNAi activities with IC_{50} values between 0.012 and 0.380 nM, which suggested that the selected siRNAs efficiently formed an RISC and degraded the artificial RLuc-oncogene fusion mRNAs.

Effects of HPV16 E6 and E7 siRNAs on endogenous mRNAs coding E6 and E7

To examine the effects of the siDirect-selected siRNAs on endogenous E6 and E7 expression, SiHa cells were transfected with the siRNAs (50 nM) for 48 h, then E6 and E7 expression was analyzed by RT–PCR. As shown in Figure 2a, PCR amplification of cDNA from control siRNA-transfected cells with a pair of E6-specific primers (E6_104s and E6_558as in Figure 1a, Table 2) gave rise to three bands representing E6, E6*I and E6*II (top panel). Transfection with siRNA 233, 243 and 244 decreased the levels of the upper band representing mRNA coding E6, but not those of the PCR bands of E6*I and E6*II. Furthermore, transfection with 493, 497 and 501 decreased the bands of E6 and E6*I, while that with 573, 698, 707 and 752 decreased all three bands. RT–PCR analysis using a pair of E7-specific primers (E7s562 and E7as855 in Figure 1a, Table 2) showed a decrease in E7 mRNA level in cells transfected with 493, 497, 501, 573, 698, 707 and 752 (middle panel). The levels of the E7 band were proportional to the sum of the E6, E6*I and E6*II levels. Each sample was also examined for β-actin mRNA level and the results were used as an internal control (bottom panel). Transfection with siRNA 497, 573, 698, 707 and 752 decreased the expression of either E6 or E7 to a level below 25%, thus they were chosen for further analyses.

Effects of HPV16 E6 and E7 siRNAs on the growth of HPV16+ and HPV16− cancer cells

HPV16+ (SiHa) and HPV16− (HeLa, SK-OV-3) cancer cells were stably transfected with a FLuc expression plasmid (hLuc/pcDNA3), and designated as FL-SiHa-2, FL-HeLa-1 and FL-SKOV-5 cells, respectively. Using an siRNA targeting FLuc mRNA (FLuc siRNA) and Lipofectamine 2000, siRNA transfection conditions for these cells were optimized to achieve comparable transfection efficiencies. Transfection with 5 nM of FLuc siRNA suppressed the FLuc activities of FL-SiH-2, FL-SKOV-5 and FL-HeLa-1 cells by 96.3 ± 0.5, 93.8 ± 0.3 and 93.3 ± 0.5%, respectively (data not shown), which demonstrated that the cells were transfected by the siRNA at similar levels of efficiency. These results enabled us to analyze the specificity of E6 and E7 siRNA-induced growth suppression.

Under the optimized transfection condition, FL-SiH-2, FL-SKOV-5 and FL-HeLa-1 cells were transfected with five different siDirect-selected siRNAs (497, 573, 698, 707, 752) as well as four others previously reported (186, 203, 222, 535)[12,14,16] and analyzed for cell viability. As shown in Figure 2b, all tested siRNAs showed moderate-to-strong growth suppression toward FL-SiHa-2 cells, while they also inhibited the growth of HPV− cells (FL-HeLa-1, FL-SKOV-5) to various degrees. Among them, 698, 186 and 535 strongly suppressed the growth of HPV16− cells, especially FL-HeLa-1 cells by more than 70%, suggesting that these siRNAs had a strong nonspecific growth inhibition characteristic.

To determine if decreased siRNA concentrations alleviated nonspecific growth suppression without compromising the specific growth inhibition, the dose effects of siRNA 497, 573, 707, 752, 203 and 222 were examined. At concentrations from 1 to 5 nM, all siRNAs suppressed the growth of FL-SiHa-2 cells in a dose-dependent manner (Figure 3c), except for 573, which exhibited similar levels of growth suppression at these concentrations. At a concentration of 1 nM, 497, 573 and 752 inhibited FL-SiHa-2 cell growth by more than 90%, while the growth suppression of HPV16− cells decreased to less than 35% (Figures 3a and b). These results suggest that 497, 573 and 752 were able to induce a potent and specific growth inhibition toward HPV16+ cells at 1 nM. In contrast, 707, 203 and 222 showed less potent growth inhibition of FL-SiHa-2 cells at 1 nM.

Dose-dependent effects of E6 and E7 siRNAs on endogenous E6–E7 mRNA

To confirm that the observed siRNA-induced growth inhibition was associated with E6 and E7 downregulation, FL-SiHa-2 cells were transfected with mock, control, E6 and E7 siRNAs (752, 203, 535) for 72 h, after which E6 and E7 expression was examined by RT–PCR using primer pairs for each (E6; 16E6s104 and 16E6as375, shown in Figure 1a, Table 2 and E7; 16E7s562 and 16E7as855). As shown in Figure 3d, siRNA 752 suppressed the level of E7 mRNA to 11, 19 and 43% at 25, 5, and 1 nM, respectively, as compared to the control siRNA-transfected cells. Furthermore, siRNA 752 decreased the E6 mRNA level to 29, 48 and 52%, respectively. In addition, two previously reported siRNAs (203, 535) showed comparable RNAi activities toward E6 and E7 mRNA. Also, 186 decreased the expression levels of both E6 and E7 mRNA, whereas 222 decreased only that of E6 (Figure 3e), which has been reported previously.[12,13] siRNA 375[14] and 660[12] were less potent and suppressed E6 and E7 expression by only 50% (data not shown).

Induction of p53 and p21$^{WAF1/CIP1}$ by E6 and E7 siRNAs

Next, we examined the downregulation of *E6* protein in the siRNA-transfected cells by analyzing the expression of

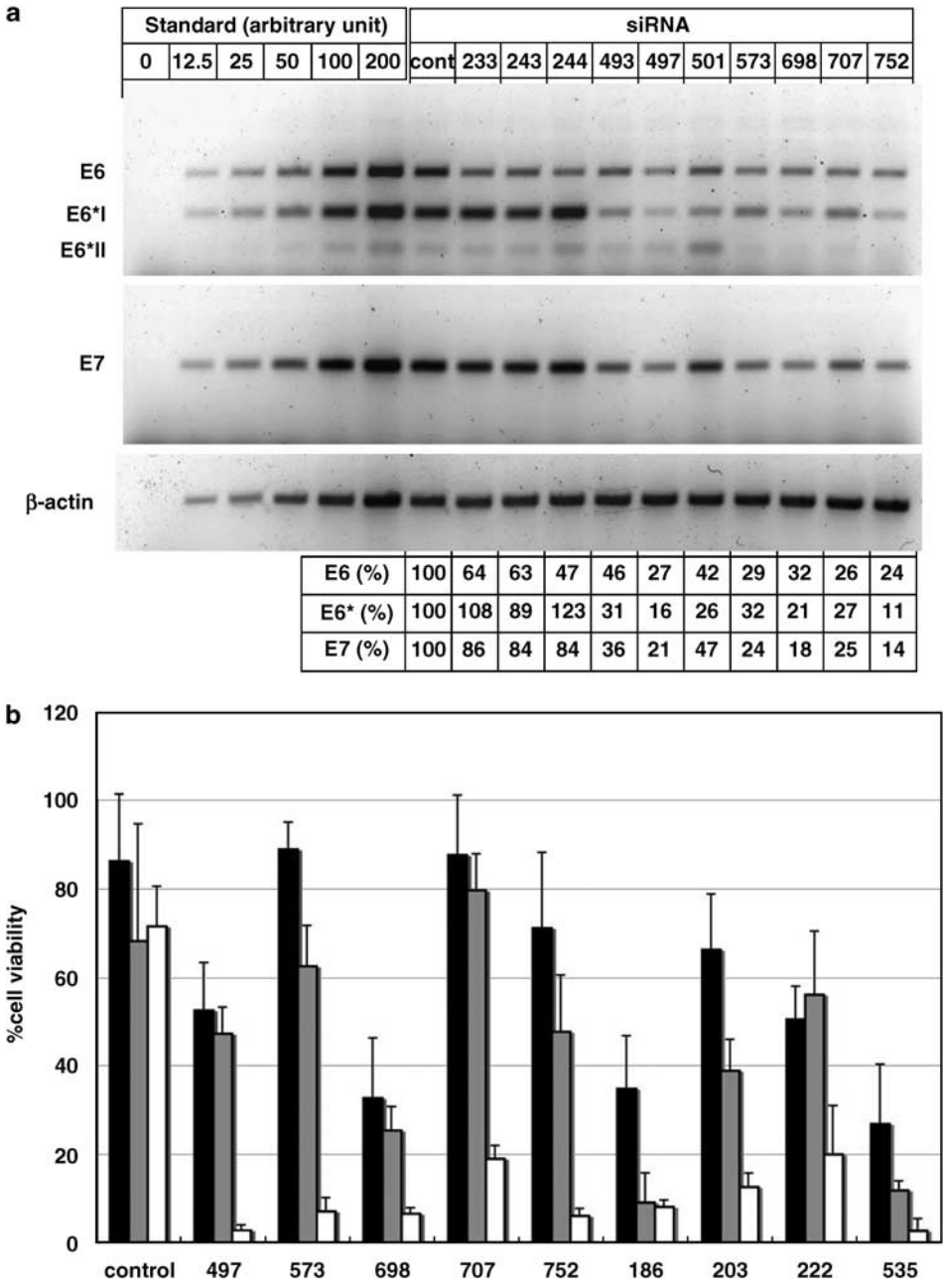


Figure 2 Effects of E6 and E7 siRNAs on E6 and E7 expression and cell growth. (**a**) Suppression of endogenous E6 and E7 expression in SiHa cells by E6 and E7 siRNAs. SiHa cells were transfected with mock, control, E6 or E7 siRNA (50 nM) for 48 h. cDNA was synthesized from total RNA and subjected to PCR. The intensity of the bands for E6 and E7 from the siRNA-treated cells were normalized to the β-actin band, and the relative E6 and E7 mRNA levels were calculated by comparing with those of serially diluted samples from mock-transfected cells. The mRNA level in control siRNA-treated cells was defined as 100%. (**b**) Effects of transfection with HPV16 E6 and E7 siRNA on growth of HPV16+ and HPV16− cells. Effects of siDirect-designed (497, 573, 698, 707, 752) and reported E6 and E7 (186, 203, 222, 535) siRNAs on the growth of HPV16− (FL-SKOV-5, FL-HeLa-1) and HPV16+ (FL-SiHa-2) cells were examined. The cells were transfected with mock, control, E6 or E7 siRNA at 5 nM, with the media changed and siRNA transfection repeated every 3 or 4 days. In 8–12 days after the first transfection, cell viability was examined using a WST-8 assay. Cell viabilities relative to mock-transfected cells are shown. Closed bar represents FL-SKOV-5; gray bar, FL-HeLa-1; open bar, FL-SiHa-2. All experiments were performed in triplicate. Error bars represent the s.d. from the mean.

p53, since there is no high affinity anti-E6 antibody capable of detecting low E6 expression presently available. p53 is an E6 target protein that degrades p53 through the ubiquitin-proteasome pathway,[2,3] while E6 knockdown restores its expression. The dose-dependent effects of siRNA 497, 573 and 752 on p53 levels were examined in FL-SiHa-2 cells using immunoblot analysis (Figures 4a–c). siRNA 497 and 573 caused a substantial accumulation of

p53 and its downstream target gene product p21$^{WAF1/CIP1}$ at concentrations from 1 to 25 nM. Transfection of siRNA 752 also caused accumulations of p53 and p21$^{WAF1/CIP1}$ at concentrations from 1 to 25 nM, with a slightly lower induction of p53 at 1 nM.

The effects of the E6 and E7 siRNAs (573, 752, 186, 222, 660, 203, 535) on the expressions of p53 and p21$^{WAF1/CIP1}$ were also examined in SiHa and CaSki cells, both of which are HPV16+ cervical cancer cell lines, using immunoblotting (Figures 4d–f). As shown in Figures 4d and e, accumulations of p53 and p21$^{WAF1/CIP1}$ were seen in SiHa cells transfected with 752, 573, 186, 222, 203 and 535, but not in those transfected with 375 and 660, which was consistent with the low RNAi activities of

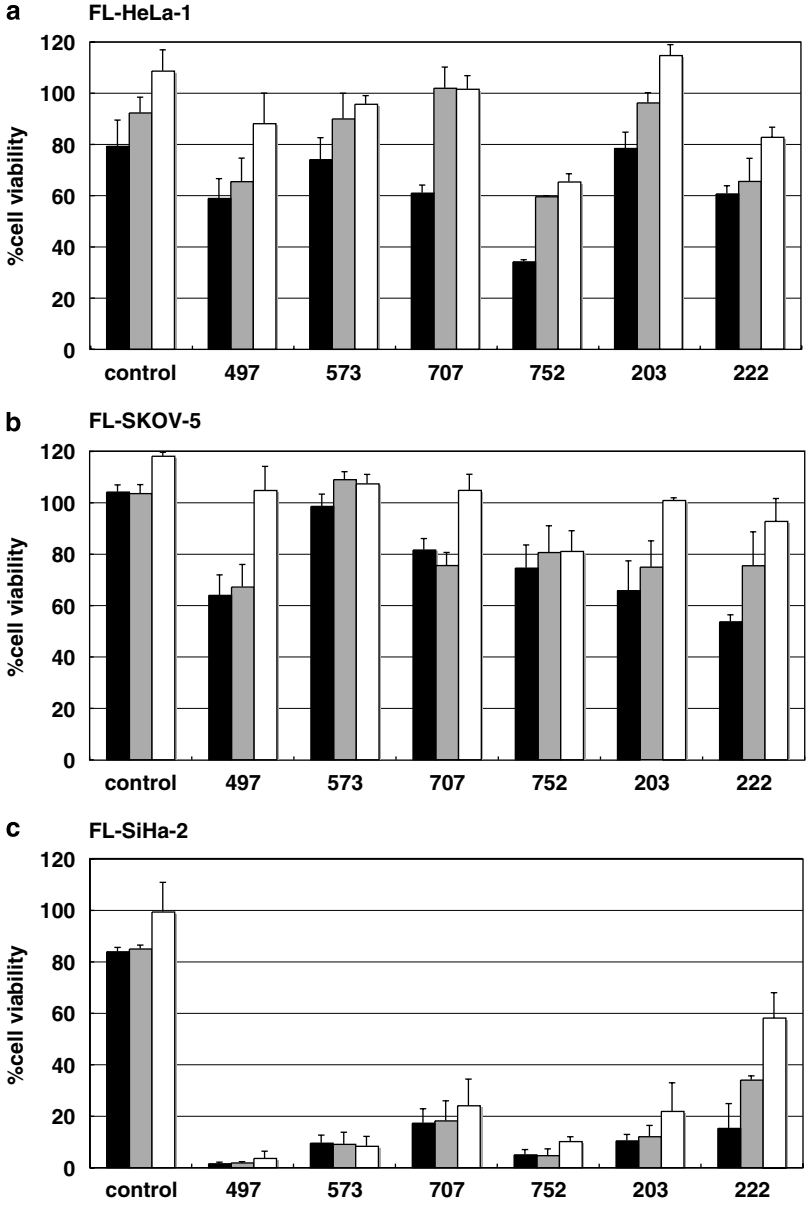
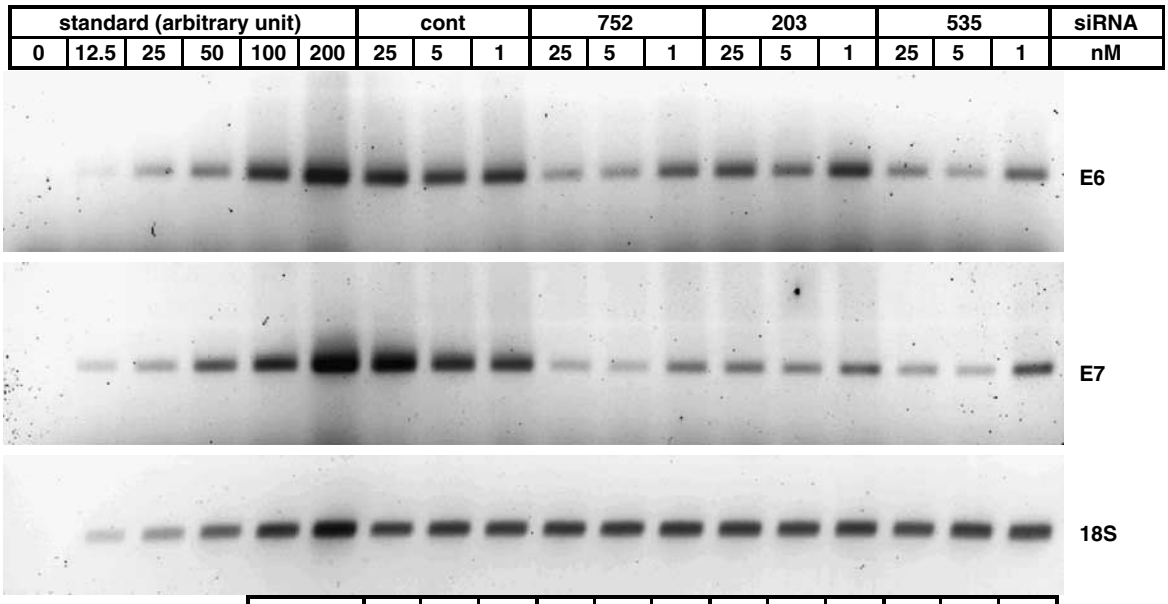


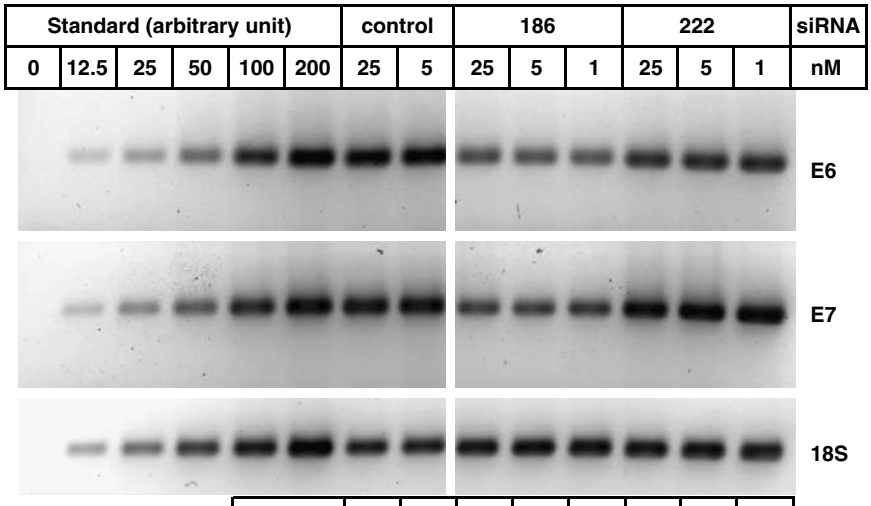
Figure 3 Dose-dependent effects of HPV16 E6 and E7 siRNAs on growth of HPV16− and HPV16+ cancer cells, and endogenous E6 and E7 expression. Dose-dependent effects of E6 and E7 siRNAs on growth of FL-HeLa-1 (**a**), FL-SKOV-6 HPV16− (**b**) and FL-SiHa-2 HPV16+ cells (**c**). Cells were plated in 96-well plates, then transfected with mock, control, E6 or E7 siRNAs (497, 573, 707, 752, 203, 222) at 5 nM (closed bar), 2 nM (gray bar) and 1 nM (open bar). Transfection was repeated every 3–4 days and a WST-8 assay was performed 7–11 days after the first transfection. Cell viabilities relative to mock-transfected cells are shown. All experiments were performed in triplicate. Error bars represent the s.d. from the mean. Dose-dependent effects of E6 and E7 siRNAs on E6 and E7 mRNA expression were analyzed in FL-SiHa-2 cells. FL-SiHa-2 cells were transfected with mock, control, E6, E7 or firefly luciferase (Fluc) siRNA at the indicated concentrations for 72 h, then analyzed for E6 and E7 expression (RT–PCR) and FLuc activity. The E6 and E7 siRNAs used in this experiment were 752, 203 and 535 (**d**), and 186 and 222 (**e**). 18S ribosomal RNA was used for normalizing the mRNA quantity. mRNA levels in siRNA-treated cells are shown relative to the level in control siRNA-treated cells. FLuc siRNA decreased FLuc activity by 96, 97 and 95% at 25, 5 and 1 nM, respectively.

d



E6	120	92	99	37	40	70	77	67	94	47	29	54
E7	153	101	87	18	17	41	42	37	44	26	21	44
18S	80	91	83	86	84	90	84	72	82	80	85	85
E6(%)	100	100	100	29	48	52	61	93	96	39	34	54
E7(%)	100	100	100	11	19	43	26	46	51	17	22	50

e



E6	174	188	70	64	63	95	106	108
E7	162	178	69	65	75	176	209	206
18S	83	88	103	124	127	118	121	102
E6(%)	100	100	32	25	24	38	42	50
E7(%)	100	100	34	27	30	76	88	103

Figure 3 Continued

those siRNAs toward E6 expression. The level of p53 level induced by 535 was disproportionate to its effect on E6 mRNA, which might have been caused by an off-target effect. CaSki cells were transfected with Oligofectamine, since Lipofectamine 2000 is cytotoxic to CaSki cells. Transfection of lamin A/C siRNA (50 nM) decreased the level of lamin A/C to 35%, suggesting that at least two-thirds of the cells were transfected with Oligofectamine (Figure 4f). The accumulation of p53 by siRNA 573 and 752 was obscure in CaSki cells as compared to SiHa cells, which was likely due to dilution of accumulated p53 by nontransfected cells containing low levels of p53.

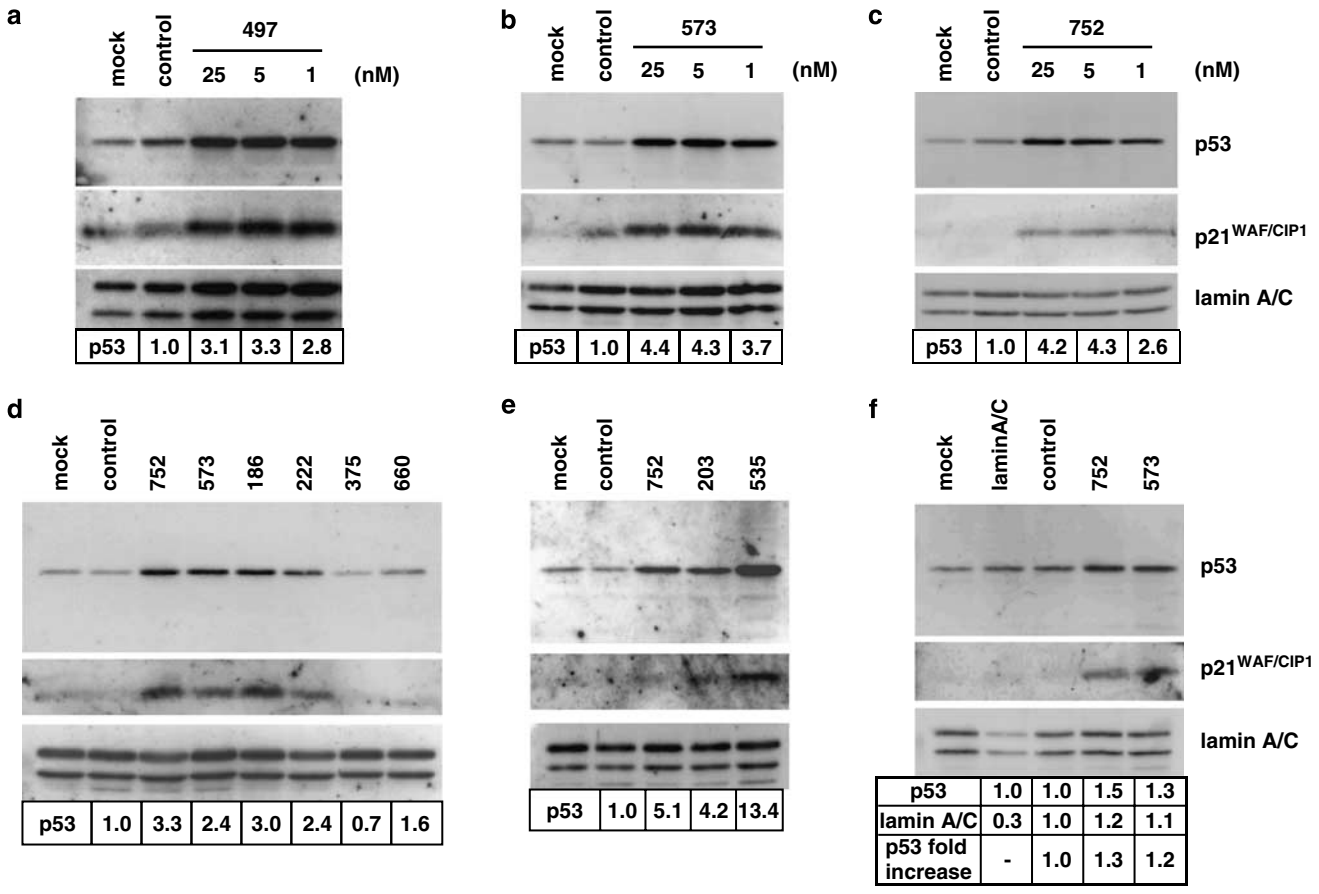


Figure 4 Effects of E6 and E7 siRNAs on p53 and p21$^{WAF1/CIP1}$ expression in HPV16+ cancer cells. FL-SiHa-2 cells were transected with mock, control, 497 (**a**), 573 (**b**) or 752 siRNA (**c**) at the indicated concentrations. SiHa cells were transfected with mock, control, 752, 573, 186, 222, 375 or 660 siRNA (**d**), or 203 or 535 siRNA (**e**) at 25 nM. CaSki cells were transfected with mock, control, 573 or 752 siRNA at 50 nM (**f**). The cells were incubated for 72 h, then analyzed for the expression of p53, p21$^{WAF1/CIP1}$ and lamin A/C by immunoblotting. p53 levels were normalized to those of lamin A/C. Lamin A/C siRNA was used to assess siRNA transfection efficiency in CaSki cells.

However, both siRNAs induced p21$^{WAF1/CIP1}$ expression in those cells, suggesting that their suppression of E6 expression caused p53 accumulation in cells effectively transfected with the siRNAs.

Induction of senescence in cervical cancer cells treated with siRNA 573 and 752

To clarify the mechanism by which E6 and E7 siRNAs induced growth suppression in cervical cancer cells, FL-SiHa-2 cells were transfected separately with siRNA 573 and 752 at 5 nM every 3 days, with cell morphological changes closely observed under an inverted microscope. Seven days after the first transfection, both 573- and 752-siRNA-transfected cells become larger and flatter, whereas mock and control siRNA-transfected cells did not change (data not shown). In 12–14 days after the first transfection, most E7 siRNA-treated cells were found to have long cytoplasmic projections and became positive for senescence associated β-galactosidase (SA β-gal) staining (Figure 5a). Furthermore, SA β-gal activity was detected in the perinuclear areas of the E7 siRNA-treated cells. These results suggested that the E7 siRNAs caused growth inhibition in HPV16+ cells by inducing cellular senescence.

Inhibition of tumor growth initiated by SiHa cells in NOD/SCID mice by siRNA 752

SiHa cells at 2×10^7 were subcutaneously inoculated into NOD/SCID mice. Six weeks after inoculation, palpable tumors had formed in all of the mice, and control or 752 siRNA complexed with atelocollagen was directly injected into each tumor every 7 days. Thirty days after the first siRNA injection, the tumors were excised and weighed. As shown in Figure 5b, tumors treated with siRNA 752 had significantly lower weights than those treated with control siRNA ($P<0.001$, mean weight: 2.73 ± 0.66 g in the control siRNA-treated group; $n=6$ versus 1.03 ± 0.29 g in the 752-treated group; $n=6$), which showed that treatment with siRNA 752 decreased *in vivo* tumor growth caused by SiHa cells.

Effects of E7 siRNAs on E7 variant expression

Three siRNAs (497, 573, 752) were chosen from 10 siDirect-selected siRNAs based on their high levels of RNAi activity and specificity. We then performed a

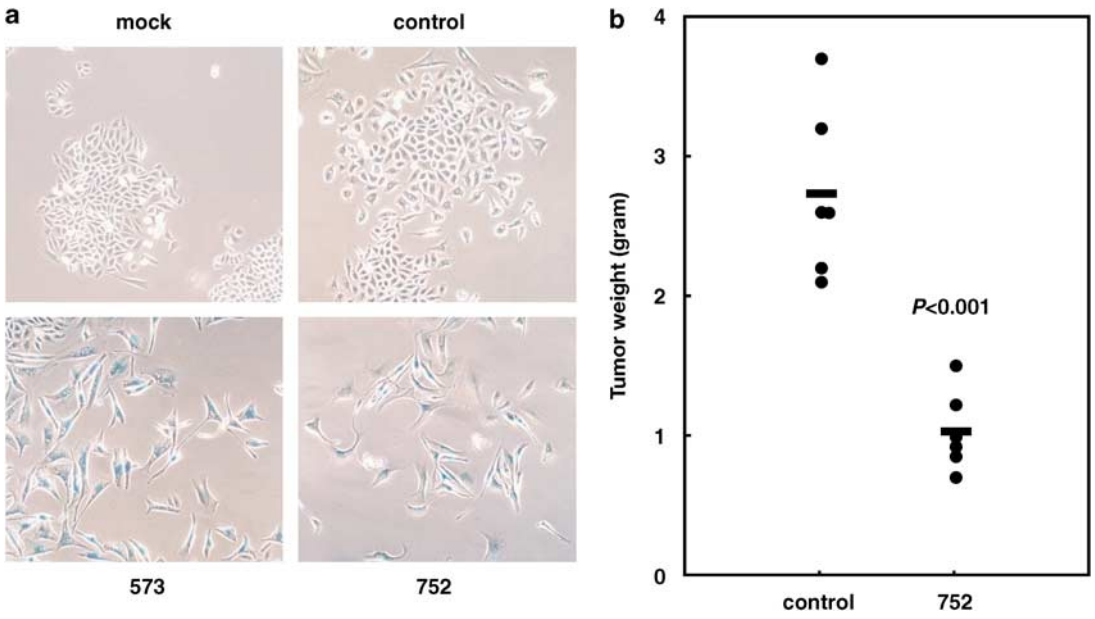


Figure 5 Induction of senescence and *in vivo* tumor growth of HPV16+ cancer cells by HPV16 E7 siRNA. (**a**) FL-SiHa-2 cells were transfected with mock, control, 573 or 752 siRNA at 5 nM using Lipofectamine 2000 every 3 days for 14 days, and then stained for senescence-associated β-galactosidase activity. Representative microscopy photographs are shown. (**b**) Suppression of *in vivo* tumor growth of SiHa cells by E7 siRNA. SiHa cells were subcutaneously inoculated into NOD/SCID mice ($n=6$). Six weeks after transplantation palpable tumors had developed, and then the control or 752 siRNA (500 pmole) complexed with atelocollagen was directly injected into the tumors every 7 days. Thirty-five days after the first siRNA injection, all tumors were excised and weighed. Bars represent the mean results ($n=6$).

literature search for E6 and E7 variant sequences that had a mismatch with these siRNAs.[32–37] A minor E7 variant, E7(760c), which contained cytosine instead of thymine at nucleotide 760,[35] was found to have a mismatch with siRNA 752. We tested the RNAi activity of 752 toward the expression of E7(760c) using a reporter gene assay. SiHa cells were transfected with the RLuc-E7(760c) fusion mRNA expression plasmid along with siRNA 752 or 752c, which was perfectly matched to E7(760C) (Supplementary Figure 1). Although both 752 and 752c suppressed the expression of RLuc-E7(760c), the 1-base mismatch decreased the IC_{50} value by fivefold (0.021 nM of 752c siRNA versus 0.107 nM of 752 siRNA). This result suggested that perfect sequence complementarity between the guide strand and target mRNA, especially in the middle portion of the guide strand, is important for RISC enzyme efficiency.

Discussion

With the aim of identifying new drug candidates for HPV16 infection and virus-related cancer, we designed siRNAs targeting HPV16 E6 and E7 oncogenes using siDirect computer software,[27] and analyzed their RNAi activities and specificity. The siDirect software is based on guidelines for the selection of effective siRNA sequences developed by Ui-Tei *et al*.[26] and enables selection of siRNAs with structural advantages for efficient loading of guide strands into RISC,[26,30,31] as well as a minimal number of off-target candidates from a database of nonredundant sequence sets of human genes. In the present study, reporter gene assays using luciferase-target fusion gene expression plasmids and our designed siRNAs revealed that all siDirect-designed siRNAs possessed high RNAi activity, as expected. However, the levels of suppression of endogenous E6-E7 mRNA in HPV16+ cancer cells varied among them. Five of the 10 siRNAs suppressed the expression of target mRNA by more than 75%, while the others showed suppression of 35–65%. These results suggest that RISC loading is not a sole determinant of RNAi activity and other factors, such as accessibility of RISC to the target sequence,[38–40] target cleavage and release of cleaved RNA, might be involved in the RNAi activities of designed siRNAs. Target accessibility is assumed to be due to the RNA secondary structure and interaction of target mRNA with other molecules. Thus, structural prediction of target mRNA would be beneficial for designing siRNAs.

Despite initial reports showing a high specificity for siRNA-directed RNAi,[10,11] recent studies have revealed that siRNAs induce off-target effects by several mechanisms.[20,25,41] An siRNA can tolerate some mismatches with its target mRNA when undergoing directed target cleavage, though enzyme efficiency decreases with increases in the number of mismatches.[25] In fact, we observed that a single nucleotide mismatch in the central portion of an siRNA targeting mRNA caused a fivefold decrease in the value of IC_{50}. siRNA sequences selected by siDirect software have at least three base mismatches with nonredundant sequence sets of human genes.[27]

Therefore, some off-target effects exerted through RISC cleavage may be attenuated by lowering the siRNA concentration.

mRNA that has a 3′ untranslated region containing a sequence complementary to nucleotide 2–7 of the 5′ end of the guide strand, analogous to the seed region of miRNA, can be a collateral target for siRNA-mediated translation inhibition.[20–22] Those studies also suggested that all siRNAs potentially possess miRNA-like activities. To minimize such off-target effects, complementarity between the guide strand and human genes should be carefully analyzed. Chemical modifications of siRNAs, such as a 2′*O*-methyl ribose modification, have been reported to reduce the miRNA-like off-target effect without compromising RNAi activity.[42]

siRNAs can also exert unintended effects through IFN-I and inflammatory cytokine production. Receptors of siRNAs for immunostimulation include double-stranded RNA-dependent protein kinase (PKR) and toll-like receptors (TLRs), such as TLR3, TLR7 and TLR8. siRNAs with sequences of 5′-GUCCUUCAA-3′, 5′-UGUGU-3′ and 5′-UGUCU-3′ have been shown to stimulate mouse TLR7 and, most likely, human TLR8.[23,24] Among the E6 and E7 siRNAs selected in our study, siRNA 497 contained the 5′-UGUCU-3′ sequence. Activation of TRLs may be bypassed by delivery of an siRNA in a cholesterol-conjugated[43] or atelocollagen-complexed form,[44] as well as following chemical modification of the RNA backbone.[45]

The mechanism by which the present siRNAs exhibited their off-target effects on HPV16− cells was not revealed. However, nonspecific growth suppression of three of the siRNAs (497, 573, 752) was significantly improved without compromising their strong growth suppression of HPV16+ cells by reducing the siRNA concentration to as low as 1 nM. To further improve the off-target effect, we are now working on backbone modification of these siRNAs, which has been reported to attenuate miRNA-like activity and cytokine response.[42,45,46]

miRNA has been implicated in diverse regulation pathways, including control of cell differentiation, apoptosis, cell proliferation and organ development. Long-term use of a high dose of siRNA might disturb these normal functions of miRNA by saturating the limited source of RNAi machinery. Therefore, it is important to administer a minimal dose of synthetic siRNA with high RNAi activity. A molecular abundance of human miRNA in HeLa cells has been reported.[47] The calculated intracellular concentrations of different miRNAs, including miR-22, miR-16, let-7 and miR-21, in HeLa cells ranges from about 0.1 to 2 nM, when assuming that the cell volume is about 10 pl. Therefore, administration of E6 and E7 siRNAs that do not exceed an intracellular concentration of 2 nM should safely exert an antitumor effect without disturbing endogenous RNAi.

In the present experiments, we identified three new siRNAs (497, 573, 752) that possessed high RNAi activities toward E6 and E7 expression, and whose growth inhibition effects were potent and specific to HPV16+ cancer cells. Furthermore, the sequences of those siRNAs showed full complementarity with all HPV16 classes and subclasses. We also performed a literature search to determine if the siRNAs were compatible with E6 and E7 variants found in patient samples from various countries (total 991 cases).[32–37] Three variants were found in the same series of patients from Hong Kong (255 patients),[35] with E6(517G), in which thymine at nt 517, located at the 5′ end of the 497 guide strand, was changed to guanine, found in three patients, E7(757T), which carried thymine instead of cytosine at nt 757, found in one patient and E7(760c), which contained thymine instead of cytosine at nt 760, found in seven patients (3.1%). These variants were not seen in patients from other areas. Thus, siRNA 497, 573 and 752 are considered to be compatible with most HPV16 variants.

Deregulation of E6 and E7 expression is a necessary cause of malignant transformation, and additional genetic alterations accumulate before progression to carcinoma development. Studies using antisense oligo-DNA, ribozymes, transcriptional suppression by forced E2 expression, and siRNAs have revealed that inhibition of E6 and E7 expression is sufficient to cause growth suppression of HPV-related cancer cells.[7–9,12–18,48] In the present study, E6 and E7 siRNA-induced growth suppression was associated with morphological and cytochemical characteristics of cellular senescence. Our results are consistent with other studies, which also demonstrated that downregulation of E6 and E7 causes *in vitro* and *in vivo* growth suppression, as well as cellular senescence.[15,16,48] Induction of senescence might be caused by reactivation of PML, which has been shown to be a mediator of senescence and a target of E7.[6] Recently, human fibroblasts immortalized by a temperature sensitive SV40 large T antigen were reported to undergo irreversible senescence by a reactivated $p16^{INK4a}$/Rb pathway after incubation at a nonpermissive temperature for a specific period of time.[49] The functional similarity between SV40 large T antigen and hrHPV E6E7 suggests that siRNA-mediated senescence might be irreversible.

Infection with hrHPV causes intraepithelial precancerous lesions in the cervix, termed cervix intraepithelial neoplasia (CIS).[50] High-grade lesions (CIS grade 3) express deregulated hrHPV oncogenes and eventually develop into invasive cervical cancer after a long latency without spontaneous regression. Downregulation of the virus oncogenes using an siRNA could reverse the neoplastic phenotype and also prevent E7-induced genetic alterations. In hrHPV-infected lesions, replication of HPVs relies on E7 expression.[5] Thus, an siRNA targeting E7 may be able to inhibit virus replication as well as propagation. CIS lesions exist within epithelium and are easily accessible.[51] Furthermore, local siRNA administration could help to avoid its hazardous systemic off-target effects. Therefore, we consider such lesions to be ideal targets of RNAi therapy.

The US Food and Drug Administration (FDA) approved a preventive HPV vaccine for immunization of women between 9 and 26 years of age, with a second

vaccine now being tested. These HPV vaccines will eventually reduce the incidence rates of viral infection and cervical cancer. However, there are currently no effective therapies for individuals infected with HPV and a small fraction of those patients will develop cancer in a decade or two.[50] Notably, immunosuppressed patients such as organ transplant recipients and HIV-infected patients, whose response to an HPV vaccine is unknown, have greater propensity for cervical cancer and anal cancer.[52,53] It is also unclear how long the protection provided by the HPV vaccines will last. Therefore, RNAi therapy using potent and specific siRNAs will benefit patients infected with HPV. Also, in parallel with HPV vaccination, RNAi therapy may speed up the reduction in rates of incidence of HPV infection and cervical cancer.

Acknowledgements

We thank Dr Hideki Matsui and Dr Kazuhito Tomizawa (Okayama University) for their helpful discussion and suggestions. This work was supported in part by Grants-in-aid for Scientific Research (C) from Japan Society for the Promotion of Science [15591749 (MY), 19592169 (KY)].

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